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Application of Polymerase Chain Reaction Based on ITS1 rDNA to Speciate *Eimeria*

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SUMMARY. A method was developed to recover *Eimeria* spp. oocysts directly from poultry litter and determine which species of *Eimeria* were present using polymerase chain reaction (PCR) based on the ITS1 rDNA sequence. The species composition of *Eimeria* oocysts was also compared before and after propagation in susceptible chickens to determine if the relative proportion of each species changed after expansion. In samples from two broiler operations, ITS1-PCR was able to detect *Eimeria* spp. oocysts recovered from litter, with *Eimeria acervulina*, *Eimeria maxima*, and *Eimeria praecox* being the predominant species present therein. Although *Eimeria tenella* was found in one sample, the other species—*Eimeria brunetti*, *Eimeria necatrix*, and *Eimeria mitis*—were not detected. The species composition as determined by ITS1-PCR did not appear to appreciably alter after expansion in susceptible chickens. The described method represents a rapid means for determining the major *Eimeria* species in a poultry operation and may be helpful in choosing a particular live oocyst vaccine formulation to protect chickens against coccidiosis.

RESUMEN. Aplicación de una prueba de reacción en cadena por la polimerasa basada en el ADN recombinante ITS1 para determinar la especie de *Eimeria*.

Se desarrolló un método para detectar oocistos de *Eimeria* spp. directamente de la cama de aves de corral y determinar cual es la especie de *Eimeria* presente mediante una prueba de reacción en cadena por la polimerasa (por sus siglas en Inglés PCR) basada en la secuencia del ADN recombinante del espaciador trascrito internamente 1 (por sus siglas en ingles ITS1). A su vez, se comparó la composición de las especies de los oocistos de *Eimeria* antes y después de la propagación en pollos susceptibles, para determinar si la proporción relativa de cada especie cambió después de la expansión. La prueba de PCR para ITS1 fue capaz de detectar oocistos de *Eimeria* spp. en la cama, siendo *E. acervulina*, *E. maxima*, y *E. praecox* las especies predominantes. Aun cuando *E. tenella* se encontró en una muestra, las otras especies *E. burnetti*, *E. necatrix*, and *E. mitis* no fueron detectadas. La composición de las especies determinadas mediante el PCR para ITS1, aparentemente no se alteró de manera apreciable con la expansión en aves susceptibles. El método descrito representa una manera rápida para la determinar las especies de *Eimeria* predominantes en una operación avícola y puede ser útil en la escogencia de una formulación particular de oocistos en una vacuna viva para proteger las aves contra la coccidiosis.

Key words: *Eimeria*, avian coccidiosis, polymerase chain reaction, detection

Abbreviations: CIA = chloroform-isoamylalcohol; E1 = expanded oocyst sample from poultry facility 1; E2 = expanded oocyst sample from poultry facility 2; ITS1 = intervening transcribed sequence 1; L1 = litter sample from poultry facility 1; L2 = litter sample from poultry facility 2; PCIA = phenol-chloroform-isoamylalcohol; PCR = polymerase chain reaction; RAPD = random amplified polymorphic DNA

Avian coccidiosis remains a significant disease problem for the U.S. and worldwide poultry industries (1,18). The use of live oocyst vaccines to prevent coccidiosis is gaining greater acceptance by broiler producers, especially with increasing drug resistance in *Eimeria* and greater consumer pressure to discontinue use of drugs in animal feed (2,17). A variety of live *Eimeria* oocyst vaccines are available, differing only by the number of *Eimeria* species present (3–7 species) and whether the parasites are virulent or attenuated (2,8,14,15). Effective application of live oocyst vaccines requires some knowledge of the species composition of *Eimeria* in a poultry facility. Although most vaccines contain the three major *Eimeria* species—*Eimeria acervulina*, *Eimeria maxima*, and *Eimeria tenella*, these vaccines would not be expected to protect chickens against infection by other species due to the lack of cross-species immunity. In general, most species of *Eimeria* oocysts cannot be distinguished from one another due to morphologic and morphometric similarities of oocysts. Several molecular techniques based on polymerase chain reaction (PCR) amplification of ribosomal DNA (e.g., ITS1, ITS2, 18S) have been developed that can discriminate between all seven species of *Eimeria* that infect chickens (6,7,10,11,12,19). A technique based on random amplified polymorphic DNA (RAPD) and markers derived from these sequences shows promise in specific amplification of *Eimeria* (3,4,5,13). While these methods are valuable in determining the species composition of highly purified oocyst samples, they have not been applied to oocysts isolated directly from litter. The present study describes a highly reproducible technique for isolating *Eimeria* oocysts from poultry litter and estimating the species composition of the oocysts therein using ITS1 PCR.

MATERIALS AND METHODS

Source of *Eimeria* oocysts. Samples of litter were collected at various locations in broiler houses from 24 different poultry farms, 12 in North Carolina (sample designation L1) and 12 in Arkansas (sample designation L2). The average age of broilers in each operation when litter was collected was 26 days. The North Carolina poultry operations were on a regular rotation of ionophore (Salinomycin), chemical/ionophore (Nicarbazin-starter/Coban-grower), and Monteban, with vaccination used for two rounds at 20 wk. The Arkansas poultry operations were on a regular rotation of ionophore (Salinomycin), chemical/ionophore (Nicarbazin-starter/Coban-grower), and Maxiban/Monteban, and no vaccines were used. The poultry were housed in standard commercial-type broiler houses equipped with nipple drinkers and standard automated feed-delivery systems. Ventilation systems in 90% of the houses were by cool cell and, in 10% of the houses, by tunnel ventilation. The litter samples were shipped overnight to the Animal Parasitic Diseases Laboratory, ARS, USDA in Beltsville, MD.

Isolation of *Eimeria* oocysts from litter. All litter samples were processed within 1 mo after delivery. Fecal droppings (~1 g each) from

Table 1. DNA sequence, annealing temperatures of ITS1 primers and predicted size of amplification products derived from ITS1-PCR amplification of *Eimeria* spp. DNA.

Eimeria species	Primer	PCR product sequence (5′–3′)	Annealing temperature (C)	Size (nt)
E. acervulina	EAF[A]	GGCTTGGATGATGTTTGCTG	60	321
	EAR[A]	CGAACGCAATAACACACGCT		
E. brunetti	EBF[A]	GATCAGTTTGAGCAAACCTTCG	45	310
	EBR[A]	TGGTCTTCCGTACGTCGGAT		
E. maxima	EMAF[B]	CGTTGTGAGAARACTGRAAGGG	51	144
	EMAR[B]	GCGGTTTCATCATCCATCATCG		
E. mitis	EMIF[C]	TATTTCCTGTCGTCGTCTCGC	54	306
	EMIR[C]	GTATGCAAGAGAGAATCGGGA		
E. necatrix	ENF[A]	TACATCCCAATCTTTGAATCG	44	285
	ENR[A]	GGCATACTAGCTTCGAGCAAC		
E. praecox	EPF[C]	CATCATCGGAATGGCTTTTTGA	54	368
	EPR[C]	AATAAATAGCGCAAAATTAAGCA		
E. tenella	ETF[A]	AATTTAGTCCATCGCAACCCT	60	271
	ETR[A]	CGAGCGCTCTGCATACGACA		

[A]*E. acervulina*, *E. brunetti*, *E. necatrix*, *E. tenella* ITS1 primer sequences as per [10].
[B]*E. maxima* ITS1 primer sequence as per [9].
[C]*E. mitis*, *E. praecox* ITS1 primer sequences as per [11].

each litter sample were removed with forceps to 50-ml polypropylene test tubes ($n = 7$). The fecal droppings were hydrated by adding deionized water to achieve a final volume of 50 ml, and then incubated for 16 hr on a rotating rocker at 4 C. The tubes were placed upright in a test tube rack, the contents allowed to settle for 3 min, and 5 ml was removed to a 15-ml polypropylene test tube containing 5 ml 2 M sucrose. The oocyst–sucrose suspension was mixed by vortexing, then overlaid with 1.0 ml deionized H_2O, and centrifuged at 2500 × *g* for

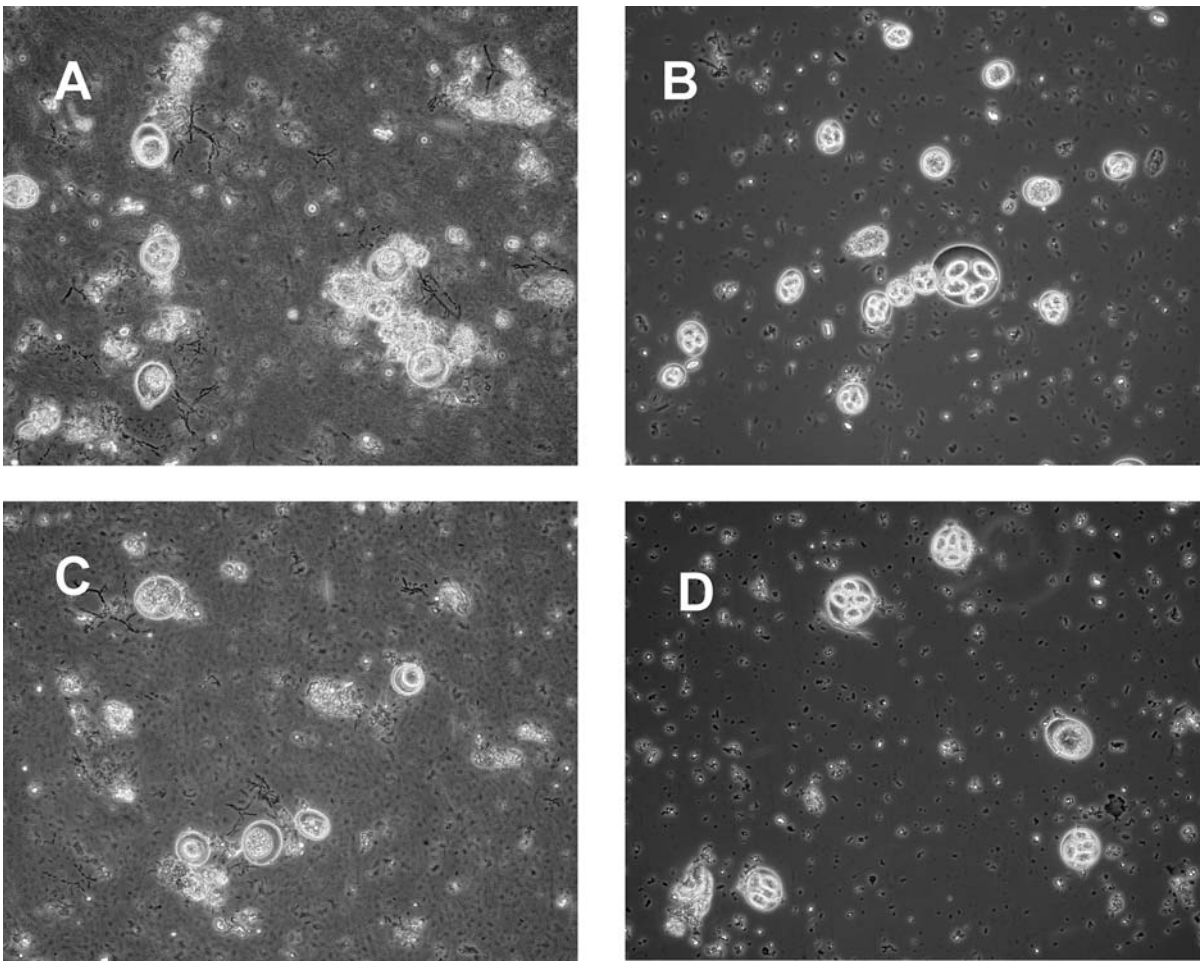

Fig. 1. Typical light microscopic view (400×) of *Eimeria* spp. oocysts recovered directly from litter (A, C) or from fecal material after propagation in susceptible chickens (B, D). (A) Litter sample (L1) from poultry facility 1, (B) expanded sample (E1) from poultry facility 1, (C) litter sample (L2) from poultry facility 2, (D) expanded sample (E2) from poultry facility 2.

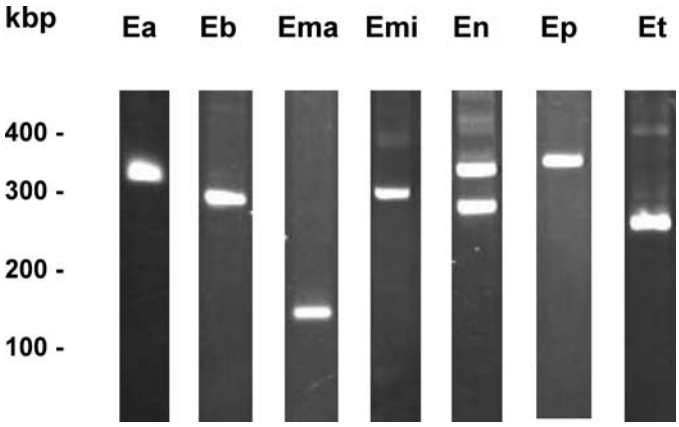

Fig. 2. PCR amplification of ITS1 ribosomal DNA from positive control *Eimeria* spp. oocyst DNA samples. Ea, *E. acervulina*; Eb, *E. brunetti*; Ema, *E. maxima*; Emi, *E. mitis*; En, *E. necatrix*; Ep, *E. praecox*; Et, *E. tenella*. kbp, 100-bp DNA markers.

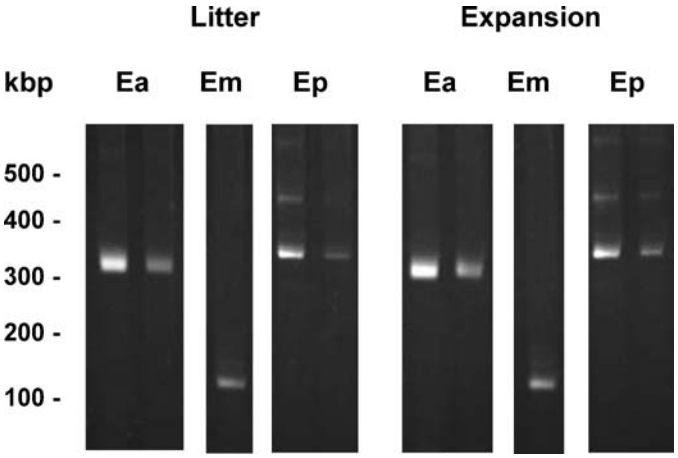

Fig. 3. Determination of species composition of *Eimeria* oocysts recovered directly from litter and from an expansion sample (by propagation in susceptible chickens) in poultry facility 1 using ITS1 PCR. Ea, *E. acervulina*; Em, *E. maxima*; Ep, *E. praecox*. kbp, 100-bp DNA markers.

10 min at 4 C. After centrifugation, 1.5 ml of the top layer was removed and mixed with 13.5 ml H_2O, pelleted by centrifugation at 2500 × *g* for 10 min at 4 C. The oocyst concentration in each tube was estimated by hemacytometer counting.

Propagation of *Eimeria* oocysts. *Eimeria* oocysts recovered from litter as described above were inoculated per os into two groups of 2-wk-old susceptible chickens ($n = 6$ chickens/group) using an animal feeding/intubation needle (VWR International, Inc., Bridgeport, NJ) at $\sim 10^5$ oocysts per inoculum. Fecal droppings were collected between days 5 and 10 and processed for total *Eimeria* oocysts using standard procedures (15). The concentration of oocysts and total number of oocysts shed in each propagation were estimated by hemacytometer counting.

Isolation of *Eimeria* oocyst DNA. *Eimeria* oocysts ($\sim 10^5$ total) were pipetted into a 2-ml screw cap tube (Fisher Scientific, Pittsburgh, PA) and pelleted by centrifugation at 8,000 × *g* for 1 min, resuspended in 500 μl of DNA extraction buffer (50 mM Tris, pH 8.0, 100 mM NaCl, 50 mM EDTA, pH 8.0), and disrupted on a Mini-Bead Beater-8 Cell Disrupter (BioSpec Products, Inc., Bartlesville, OK) apparatus using 200 mg of sterile glass beads (~0.5-mm diameter). The oocysts were broken open by four 1-min extractions, with incubation on wet ice for 1 min between each bead beating. The disrupted oocyst suspension was treated with an equal volume of phenol-chloroform-isoamylalchohol (PCIA), followed by CIA extraction, ethanol precipitation, 70% ethanol wash, drying *in vacuo,* and resuspension in 50 μl 10 mM Tris pH 8.0, 1 mM EDTA. The DNA concentration and purity was estimated by optical density ($OD_{260/280}$) reading.

ITS1-polymerase chain reaction. The *Eimeria* species present in each mixture of oocysts was determined PCR specific for the ITS1 rDNA sequence using published primer sequences (9,10,11). The optimum annealing temperatures were identified for each primer pair using positive control *Eimeria* DNA (Table 1). The reaction conditions were as follows: 1 cycle—95 C, 7 min; 35 cycles—95 C, 20 sec, 44–60 C, 30 sec, 72 C, 1 min; 1 cycle—72 C, 5 min. In each reaction (25-μl volume), an amount of total *Eimeria* DNA equivalent to approximately 2000 oocysts (~30 ηg DNA) was amplified with 25 ρmol forward and reverse ITS1 primer, 200 nM dNTP (Amersham, Piscataway, NJ), 20 mM Tris pH 8.4, 50 mM KCl, 3.0 mM $MgCl_2$, 1 U *Taq* polymerase (New England Biolabs, Ipswich, MA) in a PTC200 Minicycler™ (MJ Research, Watertown, VA). For *Eimeria* species that were present in large numbers, as indicated by an intense amplification signal, PCR was conducted on 10-fold serial dilutions of the DNA until no signal was observed. Each species-specific ITS1 PCR was conducted a minimum of three times for each litter sample. To test against false-negative reactions, PCR was also conducted on oocyst DNA using *Eimeria*-specific primers targeting the 18S ribosomal DNA sequence. The forward primer (*Eimeria* 18S-F) sequence, corresponding *E. tenella* 18S rDNA nts 78–99 was CGGTGAAACTGCGAATGGCTCA; the reverse primer (*Eimeria* 18S-R) sequence, corresponding to *E. tenella* 18s rDNA nts 411–432, was GCCTTCCTTAGATGTGGTAGCC. Aside from primer sequences, the PCR reagents and reaction conditions used in the 18S-rDNA PCR were identical to the ITS1 PCR. The amplification products were analyzed on 7.5% polyacrylamide mini-gels (BioRad Laboratories, Inc., Hercules, CA) electrophoresed at 100 volts in 40 mM Tris, 30 mM NaPO4, 1 mM EDTA, pH 7.9 buffer, using 100-bp DNA markers (BioRad) as size standards. The gels were stained with ethidium bromide and photographed on a 254-nm ultraviolet transilluminator using a CCD camera (Biophotonics, Inc., Ann Arbor, MI).

RESULTS

Processing of 7 g of fecal pellets from litter collected from poultry facility 1 produced a total of 9×10^5 *Eimeria* oocysts (sample L1). Poultry facility 1 oocysts ($\sim 10^5$) were used to produce a total of 1.05×10^8 *Eimeria* oocysts (sample E1). Likewise, processing of 7 g of fecal pellets from litter collected from poultry facility 2 produced a total of 1.3×10^6 *Eimeria* oocysts (sample L2) and a total propagation of 1.08×10^8 *Eimeria* oocysts (sample E2). The amount of DNA recovered from L1, L2, E1, and E2 oocysts was approximately 15 ρg/oocyst. A wide distribution in oocyst sizes was observed with both litter and expanded samples (Fig. 1A–D).

ITS1 PCR of positive control *Eimeria* spp. DNA samples resulted in amplification products in the expected size range (Fig. 2). Although a 285-bp product was observed with *Eimeria necatrix*-specific primers, a higher Mr band (~350 nt) was also observed. The origin of this second higher Mr band is unknown. ITS1 PCR of L1 and E1 oocyst DNA revealed the presence of three *Eimeria* species—*E. acervulina*, *E. maxima*, and *Eimeria praecox* (Fig. 3). There did not appear to be a major shift in the proportion of species between litter and expanded sample (Fig. 3). ITS1 PCR of L2 and E2 oocyst DNA revealed the presence of four *Eimeria* species—*E. acervulina*, *E. maxima*, *E. praecox*, and *E. tenella* (Fig. 4). Similar to L1 and E1, there did not appear to be a major shift in the proportion of species between litter and expanded samples (Fig. 4). Also, PCR directed at *Eimeria*-specific small subunit 18S ribosomal DNA provided evidence that the lack of a detectable signal with *Eimeria brunetti*,

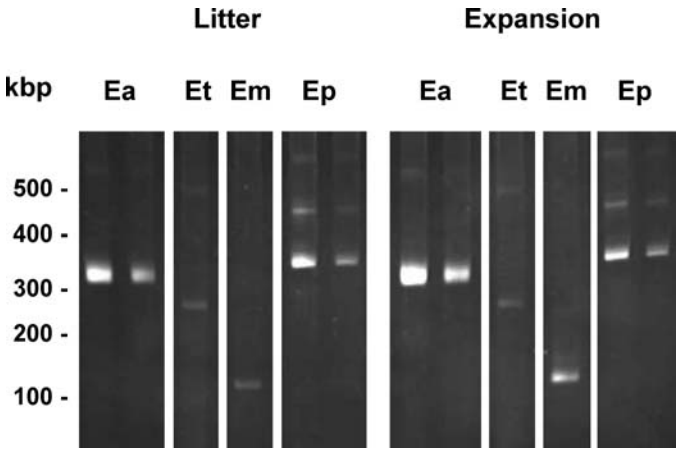

Fig. 4. Determination of species composition of *Eimeria* oocysts recovered directly from litter and from an expansion sample (by propagation in susceptible chickens) in poultry facility 2 using ITS1 PCR. Ea, *E. acervulina*; Et, *E. tenella*; Em, *E. maxima*; Ep, *E. praecox*; kbp, 100-bp DNA markers.

Eimeria mitis, and *E. necatrix* primers was not due to false-negative reactions. The 18S-rDNA target (335 nt) was amplified at all annealing temperatures using L1, E1, L2, and E2 DNA samples (Fig. 5).

DISCUSSION

The present study provides a valuable technique for isolating and speciating *Eimeria* oocysts directly from poultry litter. Although a number of molecular techniques have been used to determine the species composition of *Eimeria*, the parasite DNA in previous reports had been isolated from highly purified and concentrated oocyst samples. The yield of oocysts using the technique is probably dependent on the concentration of oocysts in litter. In this study, $1.3–1.9 \times 10^5$ *Eimeria* oocysts/g of fecal pellet was recovered from two anticoccidial drug-using broiler operations. In preliminary studies, ITS1 PCR of DNA after bead beating without further extraction did not yield a strong signal for any of the *Eimeria* species (unpubl. obs.). It appears that treatment of the disrupted oocysts suspension with phenol-chloroform, followed by ethanol precipitation, is necessary to obtain amplification of all species present. Also, bleach treatment of oocysts from litter or the expanded sample did not improve the PCR amplification signal. An important control in the described technique is the concurrent amplification of 18S rDNA using the *Eimeria*-specific 18S rDNA primers, which would test for false-negative results in the ITS1 PCR assay.

A somewhat surprising finding was the lack of a noticeable change in the species composition after propagation in susceptible chickens. *Eimeria acervulina* and *E. praecox* were the predominant species in both litter and the expanded samples. A detectable PCR product was observed for *E. maxima* in both litter and expanded samples from both broiler operations (L1, L2, E1, E2) and for *E. tenella* in litter and expanded sample from one operation (L2, E2). These results suggest that the presence of *E. acervulina* and *E. praecox* did not affect the propagation of *E. maxima* and *E. tenella*. This may not be surprising given that neither species infects the upper intestine, as observed for *E. acervulina* and *E. praecox*. It is interesting, however, that the relative amount of *E. acervulina* and *E. praecox* did not vary considerably between litter and expanded samples because these two species infect the same area of the intestine, while having slightly different patent periods (*E. praecox*, 83 hr; *E. acervulina*, 97 hr). Previous studies indicate a maximum producing dose for *E. acervulina* equal to approximately 900 oocysts, while for *E. praecox* $\leq$16 oocysts (16). Based on intensity of the amplification products from PCR of L1 and L2 DNA, about equal numbers of *E. acervulina* and *E. praecox* were present, and both were greater than both the observed crowded dose and the maximum producing dose (16). The presence of high levels of *E. praecox* and *E. acervulina* in litter, especially when the former has a higher reproductive potential than the latter (16), may be due to different sensitivities to anticoccidial drugs. One potential difficulty in using PCR to quantify the relative numbers of each species present in a sample is that there may be differences in the copy number of the ITS1-rDNA sequence between *Eimeria* species. The actual copy number of ITS1 rDNA in avian *Eimeria*, and whether it varies between species or strains, is unknown. It is also possible that several species of *Eimeria* are present in a broiler operation, but individual chickens are infected with and shedding only one *Eimeria* species at one time. Insight into the reasons for the presence of both *E. acervulina* and *E. praecox* and the concurrent expansion of all species, including *E. maxima* and *E. tenella*, after propagation in susceptible chickens will require isolation and mixing of each species for experimental infection.

The technique described will be valuable for rapidly determining the species composition of *Eimeria* oocysts in a broiler operation and should assist producers in selecting appropriate vaccines to protect against coccidiosis. One might predict that vaccines that do not contain all *Eimeria* species may allow the expansion of nonvaccine

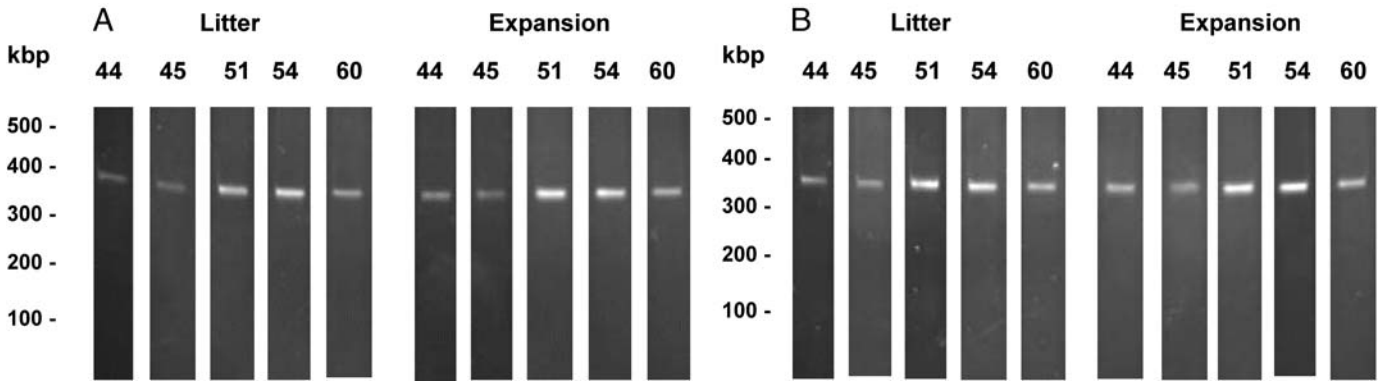

Fig. 5. Amplification of 18S rDNA sequence of DNA extracted from (A) litter (L1) or expansion (E1) or (B) litter (L2) or expansion (E2) *Eimeria* oocysts using *Eimeria*-specific primers. The numbers 44, 45, 51, 54, 60 refer to annealing temperature of PCR. kbp, 100-bp DNA markers.

strains because immunity to these is lacking. It is possible in the future that vaccines will be formulated to contain certain *Eimeria* species that are found to be present in a particular facility.

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